Population-based screening for Lynch syndrome in Western Australia

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Novelty and Impact

We report here the results of the first whole of population-based screening program for Lynch syndrome using two laboratory-based methods. Our findings indicate that approximately two thirds of all Lynch syndrome cases amongst colorectal cancer patients are being identified in the state of Western Australia. We propose that a reference laboratory for microsatellite instability testing and the position of a Lynch coordinator are critical to the success of population-based screening programs for this familial cancer syndrome.

ABSTRACT

We previously showed that routine screening for microsatellite instability (MSI) and loss of mismatch repair (MMR) protein expression in colorectal cancer (CRC) lead to the identification of previously unrecognized cases of Lynch syndrome (LS). We report here the results of screening for LS in Western Australia (WA) during 1994-2012. Immunohistochemistry (IHC) for loss of MMR protein expression was performed in routine pathology laboratories while MSI was detected in a reference molecular pathology laboratory. Information on germline mutations in MMR genes was obtained from the state's single familial cancer registry. Prior to the introduction of routine laboratory-based screening, an average of 2-3 cases of LS were diagnosed each year amongst WA CRC patients. Following the implementation of IHC and/or MSI screening for all younger (<60 years) CRC patients, this has increased to an average of 8 LS cases diagnosed annually. Based on our experience in WA, we propose three key elements for successful population-based screening of LS. First, for all younger CRC patients, reflex IHC testing should be carried out in accredited pathology services with ongoing quality control. Second, a state- or region-wide reference laboratory for MSI testing should be established to confirm abnormal or suspicious IHC test results and to exclude sporadic cases by carrying out *BRAF* mutation or *MLH1* methylation testing. Finally, a state or regional LS co-ordinator is essential to ensure that all appropriate cases identified by laboratory testing are referred to and attend a Familial Cancer Clinic for follow up and germline testing.

INTRODUCTION

Lynch syndrome (LS), formerly known as hereditary non-polyposis colorectal cancer (HNPCC), is an autosomal dominant condition caused by germline mutations in DNA mismatch repair (MMR) genes, most commonly *MLH1*, *MSH2*, *MSH6* and *PMS2*.[1,2] Tumours from LS cases have a defective DNA mismatch repair system, leading to ubiquitous small deletions and insertions in DNA repeat regions (microsatellites) resulting in microsatellite instability (MSI). MSI is almost always accompanied by the loss of expression of MMR proteins that can be readily detected using immunohistochemical (IHC) methods. These two molecular features are also observed in approximately 10% of sporadic (non-hereditary) colorectal cancer (CRC), meaning they are not completely specific markers for the presence of LS. However, sporadic microsatellite-unstable (MSI+) CRC contains mutations in the *BRAF* oncogene and often shows methylation of the *MLH1* gene promoter, whereas MSI+ CRC from LS patients does not.[3,4] Hence, the *BRAF* mutation and *MLH1* methylation tests can be used to distinguish sporadic from LS-associated MSI+ CRC.

In addition to CRC, LS is also associated with an increased risk of endometrial, small bowel, urothelial, gastric, ovarian and other cancer types. Although some authors have reported that LS may be responsible for up to 3% of CRC,[5,6] population data derived from MSI screening suggested this may be closer to 1%.[7-9, 11] Regardless of the exact prevalence, the identification of mutation carriers is critical because it allows early and increased surveillance for cancer, the option of prophylactic surgery and the ability to clarify risk status in the extended family. Regular screening by colonoscopy has been demonstrated to reduce both the incidence and mortality of CRC in LS patients and affected family members.[12] Until

recently, patients at risk for LS were identified through the use of clinical criteria such as the Amsterdam and Bethesda guidelines that rely heavily on obtaining a detailed family history.[13,14] These guidelines have a relatively low sensitivity for the detection of LS and their implementation in routine clinical practice is generally acknowledged as having been quite poor. In addition there is evidence that clinicians do not elicit a sufficient family history [15] and even when they do referrals are inappropriately low.[16] This has led to repeated calls for the introduction of a laboratory-based (MSI and IHC) screening approach that avoids the need to obtain and validate a detailed family history of cancer.[17-21]

We previously reported the results of a large retrospective study carried out in the state of Western Australia (WA) to detect LS amongst CRC patients aged <60 years at diagnosis and in the absence of any information on family cancer history.[9] This work established that MSI screening followed by testing for *BRAF* mutation in the MSI+ cases, referred to as "red flag" cases (ie. MSI+/*BRAF* wildtype), was an effective strategy for the identification of previously unrecognized LS mutation carriers in the WA population. Based on these findings and starting in 2008, routine MSI and/or IHC testing was recommended for all younger CRC patients (< 60 years) in WA, regardless of their family history of cancer.

Although the laboratory tests used to screen for MSI, MMR protein loss, *BRAF* mutation and *MLH1* methylation are not technically difficult or prohibitively expensive, their systematic introduction at a population level for the identification of LS has proven challenging. This is because of the need for cooperation and effective communication between multiple disciplines including gastroenterology, pathology, surgery, oncology and medical genetics.[21] Even greater diligence is required when the service providers are located at

different sites or work for different organizations. We present here our results on the identification of LS in the WA CRC patient cohort during an 18 year period. This includes the more recent period in which we aimed to maximize the identification of LS by implementing routine IHC and MSI testing. Based on our state-wide experience, we propose three key factors which we believe are essential for the screening of LS in entire populations, as opposed to clinic-based screening practices.

METHODS

Colorectal cancer patients

WA has a population of 2.5 million inhabitants of whom 80% live in the Perth metropolitan region. The number of newly reported cases of CRC in WA was 1,330 in 2010, the latest year for which this information was available from the Western Australian Cancer Registry.[22] Approximately 20-25% of WA CRC patients are aged <60 years at diagnosis. A public pathology service provider (PathWest Laboratory Medicine WA) with laboratories located in three teaching hospitals (Royal Perth, Sir Charles Gairdner and Fremantle hospitals) diagnoses approximately half of all CRC cases in WA. Two private pathology service providers report the large majority of the remaining cases (St John of God Pathology, Western Diagnostic Pathology). WA has a single familial cancer clinic located within Genetic Services Western Australia (GSWA) to which all suspected LS cases are referred for follow-up and germline testing. Clinico-pathological and demographic data for the CRC study cohort from 2000-2010 inclusive have been published. [9,10]

Laboratory screening tests

Over 1,300 tumours from younger (<60 years) CRC patients diagnosed in WA from 2000-2006 inclusive were tested for MSI as part of a retrospective study into population-based LS screening.[9] Based on the results of this research, WA pathology service providers agreed in 2008 to implement routine IHC screening for all newly diagnosed CRC patients meeting any of the following three criteria: (1) patients aged <60 years, (2) patients with an individual or family history of cancer, or (3) patients whose tumours have histological characteristics suggestive of LS. Since the specific screening indication was not known for all cases, these should be considered as entry guidelines rather than as specific study criteria and were not analysed separately. Prior to 2008, MSI and/or IHC testing of individual cases had been performed sporadically at the request of the surgeon and usually for younger patients or those with an individual or family history of cancer.

MSI testing was performed in the Molecular Pathology laboratory, PathWest Laboratory Medicine WA, using the pentaplex kit as described by the manufacturer (Promega, Australia). CRC patients treated at Sir Charles Gairdner and Fremantle Hospitals were tested routinely for MSI in parallel with IHC testing. Tumour samples from other WA hospitals that gave a positive result with IHC (ie. clear loss of MMR protein expression) or a result that was suspicious or difficult to interpret (clonal or heterogeneous loss of expression) were also sent to the PathWest molecular pathology laboratory for confirmation by MSI testing. All patients found to be MSI+ and with loss of MLH1 and/or PMS2 expression were subsequently screened for the presence of *BRAF* mutation as described earlier using PCR-based

techniques.[23] Patients with clear loss of MMR protein expression and with MSI+ and wildtype *BRAF* status (red flag cases) were recommended in the pathology report to attend Genetic Services WA (GSWA) for further follow-up and counselling. A database of MSI and *BRAF* testing was held by the molecular pathology reference laboratory and the results communicated by the pathologist to the treating clinician.

Follow-up of red flag cases

Treating clinicians informed red flag patients of the laboratory test result and the possible implications in terms of LS. Patients were advised to attend GSWA for counselling and possible germline testing. For patients who gave consent, germline testing for mutations in the MMR genes *MLH1*, *MSH2*, *MSH6* and *PMS2* was carried out as described previously.[9]

RESULTS

The first case of LS was identified by GSWA in 1994, shortly after pathogenic mutations in MMR genes were identified as the cause of what was then known as HNPCC. Over the ensuing 11 years until 2004, from 1-4 new cases were identified each year (Figure 1). This was recognized at the time as being less than expected, considering that the annual incidence of CRC in WA was approximately 1,000 cases during this period [22] and assuming that LS is responsible for 1-3% of all CRC.

Beginning in 2003, retrospective screening of archival CRC tissues using MSI testing was performed in an attempt to identify previously unrecognized LS cases. The first pilot study involved the screening of 1,020 CRC diagnosed at a single hospital (Sir Charles

Gairdner Hospital) from 1990-1999 inclusive.[24] The second study involved the screening of almost 1,400 CRC patients aged <60 years who were diagnosed from 2000-2007 inclusive.[9] This was estimated to have captured 85% of the state's CRC cases in that age group reported to the WA Cancer Registry during the 7 year study period. Red flag cases (MSI+, *BRAF* wildtype) identified by these two studies were followed up and offered genetic testing, resulting in a spike in the number of LS cases diagnosed during 2005-2007 (Figure 1). During this 3 year period 44 new LS cases were identified, some of whom had been diagnosed with CRC up to 12 years earlier.

At the completion of the retrospective screening projects [9,24] and based partly on the results obtained from this research, routine IHC and/or MSI screening was begun in 2008 by all the major pathology service providers in WA. Since then, the number of newly identified LS cases has been remarkably constant at 8, 10, 8, 8 and 8 per year (mean = 8.4; Figure 1). If LS cases identified by retrospective screening of younger CRC patients diagnosed from 2000-2007 are included, the total number identified since 2000 is 103, giving an annual diagnosis rate of 7.9 (103/13).

To the end of 2012, 115 unrelated LS families with MMR gene mutations had been identified by GSWA, consisting of 40 *MLH1*, 51 *MSH2*, 11 *MSH6* and 9 *PMS2* germline mutations. In addition, the retrospective MSI screening of CRC identified 3 families with epigenetic mutations in *MLH1* and one family with an *EPCAM* germline mutation. One of the families with an *MLH1* epimutation has been described elsewhere.[25] A further 177 mutation positive family members had also been identified by predictive testing (Figure 2), giving a total of 292 mutation carriers identified thus far in WA.

Compared to the InSiGHT database of 3,072 reported MMR gene mutations,[26] the WA LS cohort displayed a significantly higher proportion of *MSH2* mutations (*P*=0.005) but a lower proportion of *MSH6* mutations (*P*=0.03) (Figure 3). No significant differences were apparent for the relative frequencies of *MLH1* and *PMS2* mutations between these two cohorts.

DISCUSSION

There have been numerous reports on the use of MSI and IHC screening for the detection of LS in CRC cohorts.[2,6,7,8,11,27] The present study follows on from our earlier screening of retrospective CRC cases [9,24] and is to our knowledge the first to use laboratory tests on a whole of population basis. Whereas other groups have drawn their study cohort from a single or small number of institutes with ill-defined catchment areas and with possible referral bias, we investigated the entire CRC cohort from a defined geographic region. This has allowed us to estimate the detection rate for LS in the state of WA. WA is an isolated state with a population of 2.5 million inhabitants and a CRC incidence of approximately 1,400 cases per year.[22] This population is served by a single familial cancer clinic run within the public health sector and which maintains a database of LS cases at the state level. Since the first reports linking LS with hereditary MMR gene mutations in the early 1990's, the identification in WA of individuals with this familial cancer condition has occurred in three phases (Figure 1). The first from 1994-2004 relied mainly on use of the Amsterdam and Bethesda criteria, the second from 2005-2007 involved research studies using MSI testing on retrospective cases, while the third phase since 2008 has involved the implementation of routine IHC and centralised MSI

screening. The incidence of CRC in WA rose from 1,053 cases in 2000 to 1,330 in 2010, due to the increase in, and ageing of the population.[22] However, this increase in CRC incidence cannot account for the more than 3-fold increase in the rate of LS diagnosis between the first and third phases (2.6 *vs* 8.4 cases per year). Many of the screen-detected LS patients did not report a family history of cancer sufficient to meet the Amsterdam or Bethesda criteria and hence it is unlikely they would have been identified without laboratory screening tests. We conclude from our results that routine MSI and IHC screening of CRC has led to a marked increase in the identification of LS in WA.

We cannot be sure that all the LS cases amongst WA CRC patients are now being detected following the implementation of routine IHC and MSI screening. This is because of the uncertainty surrounding the contribution made by LS to the overall incidence of CRC. Hampel *et al* reported a prevalence of 2.8% for LS in their Ohio University study of 1,566 CRC patients.[5] A pooled analysis of 4 large cohorts that included the Ohio series reported an MMR gene mutation frequency of 3.1%.[6] However, two other large MSI-based screening studies comprising more than 1,000 CRC patients each reported frequencies of 0.86% [7] and 0.9%,[8] consistent with our earlier estimate of 0.83%.[9] A recent clinic-based study estimated the germline mutation frequency amongst 245 consecutive cases of MMR deficient CRC was 7.1%.[11] Since the incidence of MMR deficient tumours in their overall CRC cohort was 11.7%, this implies the prevalence of LS was 0.83%. If we therefore assume that 0.8-0.9% is an accurate estimate of the contribution of LS to CRC in the WA population, we could expect to find approximately 12 new LS cases per year in this state (0.9% x 1,300). This compares with the observed detection rate of 7.9 cases per year since 2000 (103 cases/13 years), spanning both the retrospective and prospective screening periods.

There are likely to be several reasons as to why only two thirds of the 12 expected annual LS cases are being identified, even with the introduction of laboratory-based screening. Many of these factors were also highlighted in a recent clinic-based study.[27] First, only CRC patients aged <60 years were screened for MSI in the retrospective study of 2000-2006 cases.[9] Although the large majority of LS patients are diagnosed with CRC at an early age (mean of 43 years in our cohort), there are certainly older patients who would not be detected using an age threshold of <60 years for screening. Second, we cannot be certain that all pathology service providers, particularly those with lower case volumes, have carried out the IHC test on all appropriate cases, as defined in the Methods. Third, there are currently no local quality control procedures in place to ensure the accuracy of IHC testing. Pathologists who observe heterogeneous or ambiguous staining patterns are encouraged to submit tumour samples to the state's molecular pathology reference laboratory for MSI testing. Fourth, a small number of red flag cases were not referred by their clinician to GSWA, some were referred but did not attend, some died prior to attending and others attended but did not give consent for germline testing. The proportion of red flag cases who gave consent for germline testing during the 2011/2012 period (81%, 26/32) and in the earlier 2009/2010 period (88%, 38/43) [10] was considerably higher than that reported in another recent Australian study (46%, 112/245). [11] This may be attributable to greater awareness by treating clinicians of the need to refer red flag cases to GSWA, as well as the presence of an LS coordinator in the state of Western Australia. Finally, there are likely to be technical limitations with the identification of some germline mutations and uncertainty as to whether some genetic variants considered as "unclassified" may indeed be pathogenic.

Several of the above limitations can be readily addressed and should result in an increased rate of detection at minimal cost. Regular auditing by the treating clinician would ensure that all appropriate CRC cases are tested by IHC and/or MSI. Quality control measures could also be introduced to ensure the accuracy of laboratory results. Further improvements in communication between pathologists, gastroenterologists, surgeons and patients would ensure that all red flag cases are referred to GSWA in a timely manner. Since 2009, a minority of red flag patients (15%, 11/75) had contact with the Familial Cancer Clinic at GSWA but declined germline testing for various reasons including ongoing adjuvant treatment, lack of interest and residence in rural or remote areas of the state.

Based on our experience with population-based screening in WA, we propose three key elements that are critical for successful implementation of LS screening in other states or regions (Figure 4). First, reflex IHC testing should be carried out in accredited pathology services with ongoing quality control systems. This should be performed for all younger (<60 years) CRC patients, those with an individual and/or family history of cancer suggestive of LS, and patients whose tumours have histological characteristics suggestive of LS. Some centres undertake reflex testing of all CRC cases. While this approach may increase the detection of LS in older patients, it must be weighed up against the increased cost of testing.

Second, a state- or region-wide reference laboratory for MSI testing is required to confirm all abnormal or equivocal IHC test results identified in the first screen by pathology service providers. This is critical in order to avoid large numbers of cases with false positive IHC results from being referred to familial cancer clinics for follow up. The quality of IHC testing and its interpretation vary between laboratories and this may be due to recognized

factors such as tissue fixation and heterogeneity of staining.[28] Compared to IHC, the MSI test is in our experience less prone to errors of interpretation and is more readily standardized. In addition to MSI testing, the reference molecular pathology laboratory should be capable of performing *BRAF* mutation and/or *MLH1* methylation assays so that sporadic MSI+ cases can be excluded,[3,4] again preventing many unnecessary referrals to familial cancer clinics. Although IHC for the detection of *BRAF* mutations has been suggested, a recent study found this approach cannot be used as a surrogate to genotyping due to inadequate sensitivity. [29]

The third critical element is the existence of a state- or region-wide LS coordinator to ensure that all red flag cases identified by laboratory testing are referred to a familial cancer clinic in a timely manner, prior to the patient becoming too ill or even dying. This increases the likelihood of collecting blood before the start of chemotherapy. The position of LS coordinator would ideally be embedded within a genetic service, as in WA, or alternatively hosted by an independent organization such as a state or regional health department, or a state cancer council. The coordinator must have direct and regular communication with the molecular pathology reference laboratory and maintain a database of red flag cases for their catchment area. This allows monitoring of both referrals and attendance at familial cancer clinics. A recent study by the Cleveland Clinic demonstrated that direct contact of patients with MSI/IHC abnormalities (ie. red flag cases) by a genetic counsellor was an efficient means of ensuring attendance at a familial cancer clinic.[27] At present in WA, only a small number of red flag cases are not being referred by clinicians to GSWA (approximately 6/38 per year, 16%).

Although the scheme outlined in Figure 4 for population screening of LS has led to good outcomes in WA, several issues require further investigation. First, what is the cost-effectiveness of laboratory-based screening for LS? This has been investigated for single institutions, [30,31] but not for population-wide screening as proposed here. The benefits of the scheme proposed in Figure 4 cannot be quantified until a full cost-benefit analysis has been performed. Second, do LS cases identified by population-based laboratory screening have a different phenotype and genotype to those identified in familial cancer clinics using the Amsterdam and Bethesda criteria? The significantly different proportions of *MSH2* and *MSH6* mutations observed in the WA LS cohort compared to the InSiGHT database (Figure 3) suggests this may be the case. Finally, would routine IHC and/or MSI screening of endometrial cancers and other LS-related cancers, particularly in younger patients, identify previously unrecognized cases of LS?

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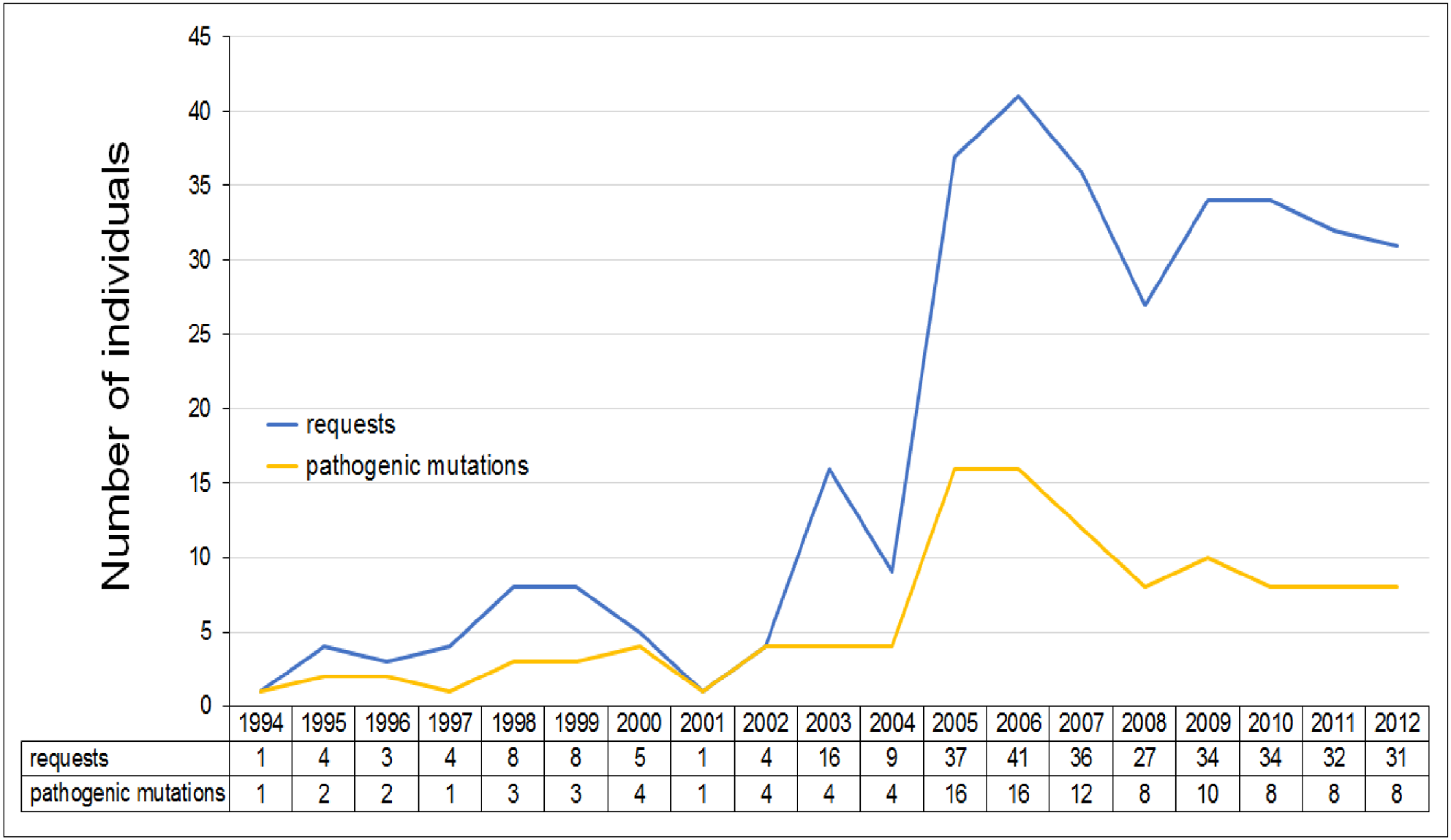
FIGURE LEGENDS

Figure 1. Diagnostic genetic testing for Lynch syndrome in Western Australia, 1994-2012. The first period from 1994-2004 involved mainly the use of Amsterdam and Bethesda criteria, the second period from 2005-2007 involved laboratory-based screening of retrospective cases, while the most recent period since 2008 has involved routine, prospective testing with IHC and MSI.

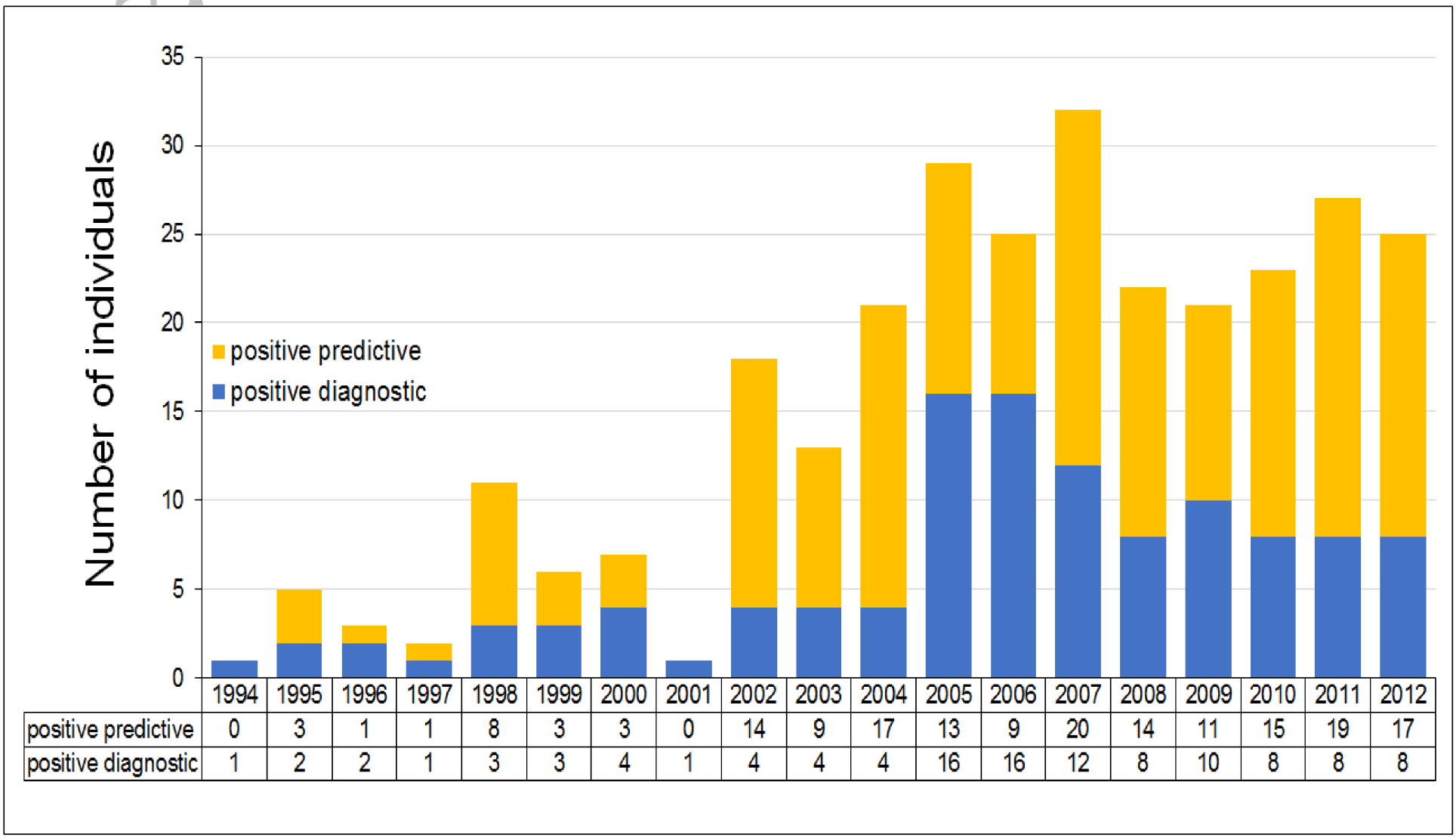
Figure 2. Mismatch repair gene mutation carriers identified through diagnostic and predictive testing in Western Australia, 1994-2012. The total number of index cases identified to the end of 2012 is 115. Predictive testing of family members has identified a further 177 mutation carriers, giving a total of 292 individuals with germline mutations for MMR genes.

Figure 3. Comparison of germline mutations in MMR genes between the WA LS cohort (blue) and mutations reported to the InSiGHT database (yellow). A significantly higher proportion of mutations in *MSH2* and *MSH6* were found in the WA cohort.

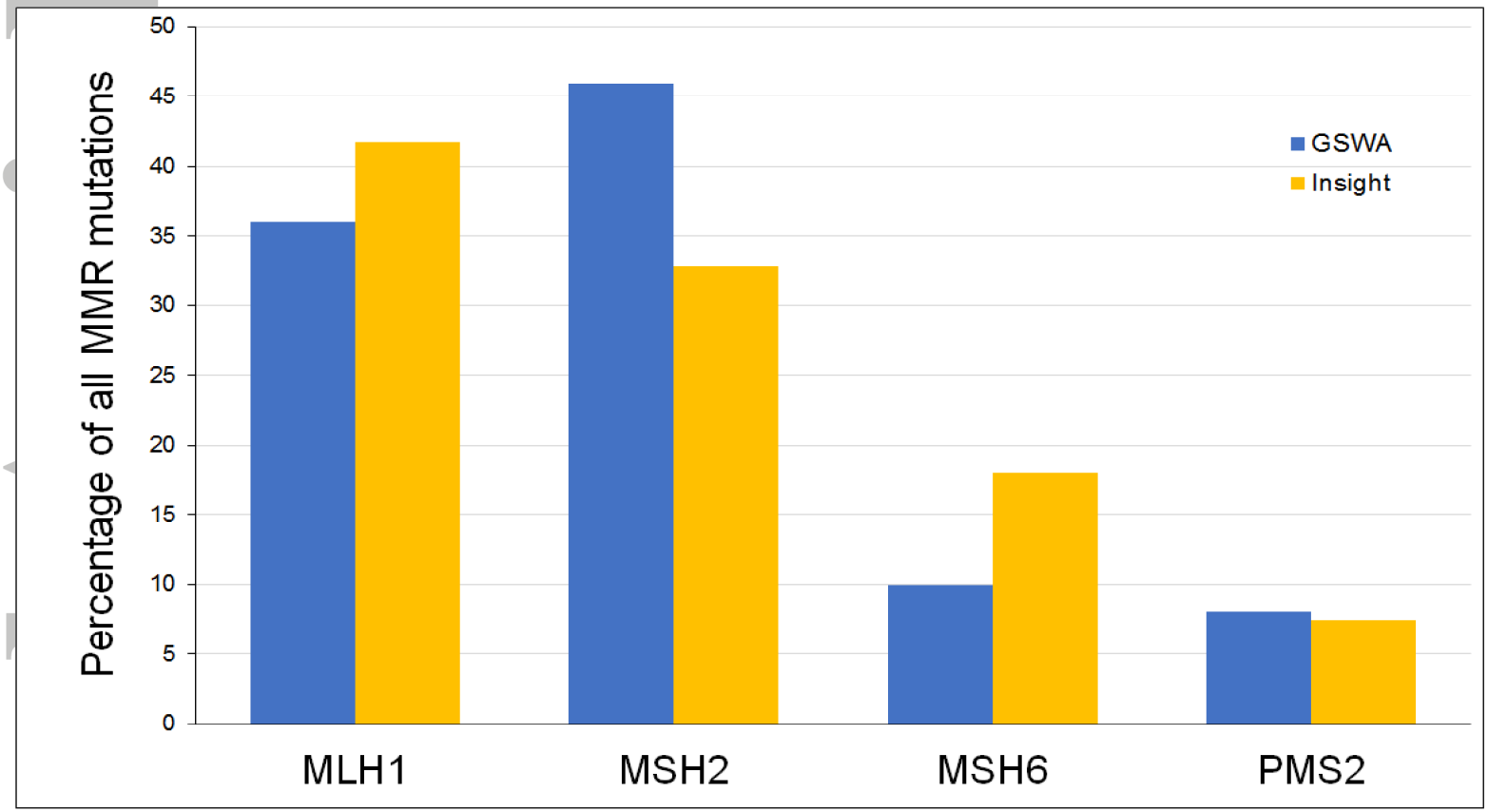
Figure 4. Proposed flowchart for population-based screening of Lynch syndrome in CRC patients. The three key elements of this scheme are reflex IHC testing, a centralized molecular pathology laboratory for MSI and *BRAF* mutation/MLH1 methylation testing, and a state- or region-wide LS coordinator.

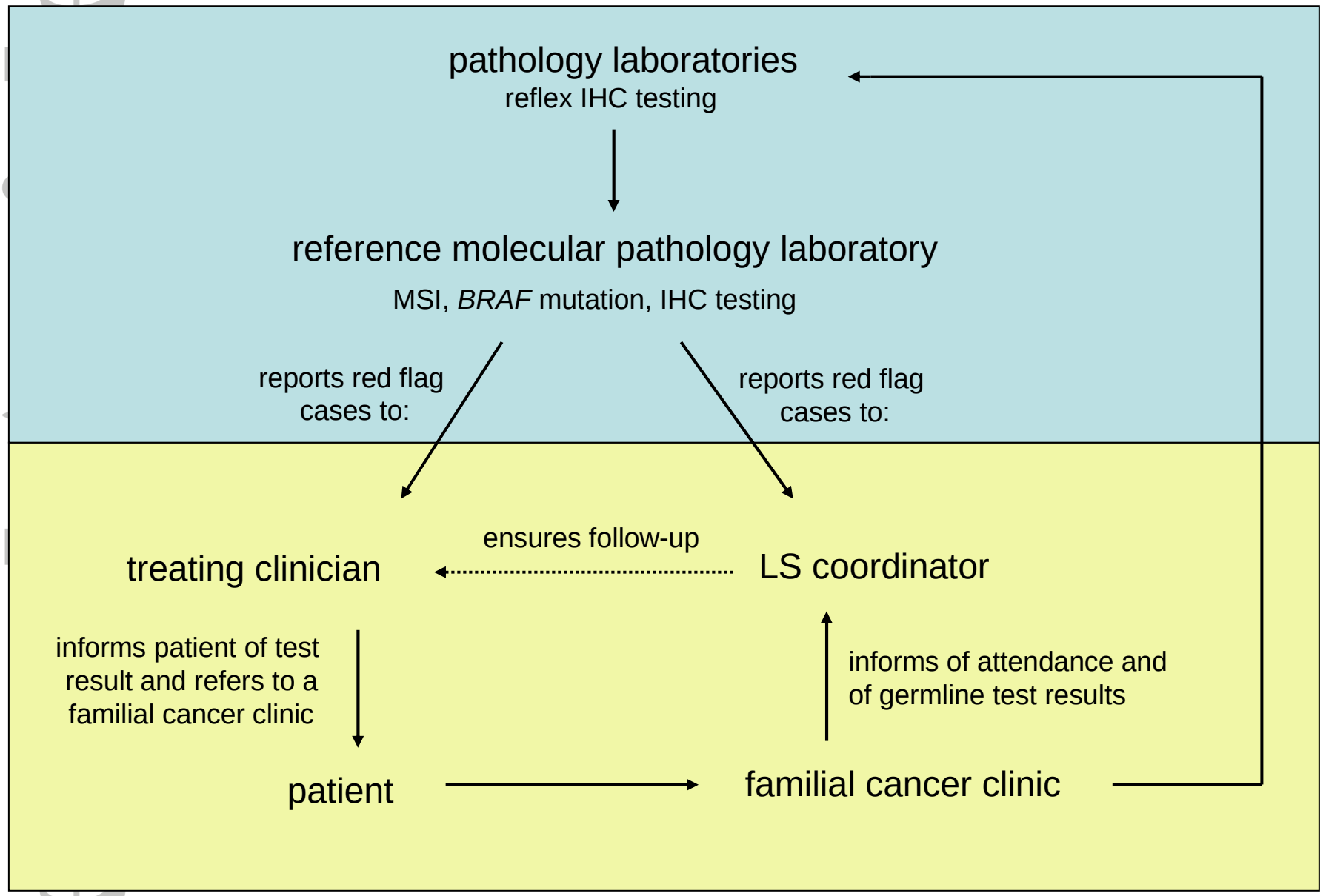


	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
requests	1	4	3	4	8	8	5	1	4	16	9	37	41	36	27	34	34	32	31
pathogenic mutations	1	2	2	1	3	3	4	1	4	4	4	16	16	12	8	10	8	8	8



	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
positive predictive	0	3	1	1	8	3	3	0	14	9	17	13	9	20	14	11	15	19	17
positive diagnostic	1	2	2	1	3	3	4	1	4	4	4	16	16	12	8	10	8	8	8




pathology laboratories
reflex IHC testing
reference molecular pathology laboratory
MSI, *BRAF* mutation, IHC testing
reports red flag cases to:
reports red flag cases to:
ensures follow-up
treating clinician
LS coordinator
informs patient of test result and refers to a familial cancer clinic
informs of attendance and of germline test results
patient
familial cancer clinic